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Video Article

Use of Human Perivascular Stem Cells for Bone Regeneration

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Abstract

Human perivascular stem cells (PSCs) can be isolated in sufficient numbers from multiple tissues for purposes of skeletal tissue engineering¹⁻³. PSCs are a FACS-sorted population of 'pericytes' (CD146+CD34-CD45-) and 'adventitial cells' (CD146-CD34+CD45-), each of which we have previously reported to have properties of mesenchymal stem cells. PSCs, like MSCs, are able to undergo osteogenic differentiation, as well as secrete pro-osteogenic cytokines^{1,2}. In the present protocol, we demonstrate the osteogenicity of PSCs in several animal models including a muscle pouch implantation in SCID (severe combined immunodeficient) mice, a SCID mouse calvarial defect and a femoral segmental defect (FSD) in athymic rats. The thigh muscle pouch model is used to assess ectopic bone formation. Calvarial defects are centered on the parietal bone and are standardly 4 mm in diameter (critically sized)⁸. FSDs are bicortical and are stabilized with a polyethylene bar and K-wires⁴. The FSD described is also a critical size defect, which does not significantly heal on its own⁴. In contrast, if stem cells or growth factors are added to the defect site, significant bone regeneration can be appreciated. The overall goal of PSC xenografting is to demonstrate the osteogenic capability of this cell type in both ectopic and orthotopic bone regeneration models.

Video Link

The video component of this article can be found at <http://www.jove.com/video/2952/>

Protocol

1. Perivascular Stem Cell Isolation

This is described in details in the adjacent article "Purification of Perivascular Stem Cells from Human White Adipose tissue", by M. Corselli *et al.*

2. Scaffold Creation

1. Scaffolds are custom-made per previously published protocol from poly(lactic-co-glycolic acid) (PLGA, Burmingham Polymer) with hydroxyapatite coating⁴⁻⁶. Apatite-coated PLGA scaffolds are fabricated from 85/15 PLGA by solvent casting and a particulate leaching process. Scaffolds are created in a spherical shape (2-mm diameter) for muscle pouch implantation, a discoid shape (4 mm in diameter) for calvarial implantation, or cylindrical (4 mm in diameter, 6 mm in length) for femoral segmental defects.
2. Briefly, PLGA/chloroform solutions mixed with sucrose (polymer/sucrose ratio 5/95, w/w) are cast into a 200-300-µm diameter Teflon mold to create the custom-made construct. After freeze-drying overnight, scaffolds are removed from the Teflon mold and immersed in ddH₂O to dissolve the sucrose. Scaffolds are disinfected by immersion in 70% ethanol for 30 min, followed by three rinses of ddH₂O.
3. For apatite coating, a simulated body fluid (SBF) solution is prepared by sequentially dissolving CaCl₂, MgCl₂·6H₂O, NaHCO₃, and K₂HPO₄·3H₂O in ddH₂O. Solution pH is lowered to 6 by adding 1M hydrochloric acid to increase the solubility. Na₂SO₄, KCl, and NaCl are added and the final pH is adjusted to 6.5 (SBF 1).
4. Mg²⁺ and HCO₃⁻ free SBF (SBF 2) is prepared by adding CaCl₂ and K₂HPO₄·3H₂O in ddH₂O and pH is lowered to 6. KCl and NaCl are added and the final pH is adjusted to 6.8. All solutions are sterile filtered through a 0.22 µm PES membrane (Nalgene). Immediately prior to the coating process, the dried PLGA scaffolds are subjected to glow discharge argon plasma etching (Harrick Scientific) to improve wetting and coating uniformity.
5. Etched scaffolds are then incubated in SBF 1 for 12 h and changed to Mg²⁺ and HCO₃⁻ free SBF 2 for another 12 h at 37 °C under gentle stirring. Coated scaffolds are washed with ddH₂O to remove excess ions and lyophilized prior to further studies.

3. The Muscle Pouch Model Implantation

1. 100 μ l of a PSC suspension in PBS (phosphate buffered saline) are gently dropped onto the spherical PLGA-based implant immediately prior to implantation. Cells have been pre-labeled by lentiviral insertion of Firefly Luciferase, so as to permit in vivo tracking post implantation. Cell density is 2.5×10^5 per implant.
2. SCID (severe combined immunodeficient) mice are used at 6 weeks of age. Animals are anesthetized by isoflurane inhalation and premedicated with buprenorphine (Bedford Labs). After standard Betadine preparation, bilateral incisions in the hindlimbs are made (longitudinal and 2 mm in length).
3. Pockets are cut in the biceps femoris muscles by blunt dissection parallel to the muscle fiber long axis. For each mouse, the PLGA-based implant with PSCs is inserted, and the fascia overlying the muscle is sutured with 5-0 Vicryl (Ethicon).
4. The skin is next closed with 5-0 Vicryl in a subcuticular pattern. Animals are treated postoperatively with buprenorphine for 48 hours and TMP/SMX (Trimethoprim/ Sulfamethoxazole; Qualitest) for 10 days.

4. The Calvarial Defect Model Implantation

1. After isoflurane anesthesia of SCID mice (12-14 weeks old), the hair is clipped and the skin disinfected with betadine per protocol.
2. A 8-mm skin incision is made along the mid-sagittal suture of the mouse calvarium. Next, the calvarial periosteum is gently removed by Q-tip application.
3. Next, using diamond-coated trephine bits in a high-speed dental drill, a 4-mm parietal bone defect is created. The defect is full thickness - however care is taken not to injure the underlying dura mater.
4. The custom-made PLGA-based implant with engrafted PSCs is then placed gently in the defect site. Cell density is 2.5×10^5 per implant. Finally, the skin is sutured with 6-0 Vicryl. Animals are treated postoperatively with buprenorphine for 48 hours and TMP/SMX for 10 days.

5. The Femoral Segmental Defect Model Implantation

1. Athymic rats (12-14 weeks old) are anesthetized under isoflurane inhalation. The femur is scrubbed and prepared per standard protocol with betadine (**Figure 1**).
2. A 27-30-mm longitudinal incision is made over the anterolateral aspect of the femur. The femoral shaft is then exposed by separating the vastus lateralis and biceps femoris muscles (**Figure 2**).
3. To maximize consistency of bone regeneration, the periosteum overlying the femoral defect is completely removed with the resected femoral segment.
4. A polyethylene plate (length, 23 mm; width, 4 mm; height, 4 mm) is placed on the anterolateral surface of the femur. The plate contains six pre-drilled holes to accommodate 0.9 mm diameter threaded Kirschner wires (Zimmer). Taking the plate as a template, six threaded Kirschner wires are drilled through the plate and both cortices (**Figure 3**).
5. Next, with a small oscillating saw blade (Stryker, MI), a 6-mm mid-diaphyseal defect is created. Segmental defects are then treated by the insertion of PLGA based implants which have been laden with cells as per protocol (**Figure 4**).
6. The overlying muscle and fascia are closed with 4-0 Vicryl absorbable sutures to secure the implant in place, and the skin is sutured.

6. In Vivo Assessments

1. Radiographic assessments are performed in a longitudinal manner by both high resolution XR and high resolution μ CT (micro computed tomography) analysis. For μ CT analysis (Skyscan 1172F), images are scanned at a resolution of 19.73 μ m (100 kV and 100 mA radiation source, using a 0.5 mm aluminum filter). Images are analyzed using DataViewer, Recon, CTAn, and CTVol softwares.
2. Bioluminescence imaging is also done in a serial manner to assess cell engraftment, viability, proliferation and exclude migration out of the implant site. Bioluminescence imaging is performed using an IVIS Lumina II device (Caliper Life Sciences). Light outputs are quantified using Living Image software (Xenogen). Total light output is recorded in photons/second/sq cm/steradian.
3. Histological and histomorphometric analysis is performed postmortem. Routine stains employed include Masson's trichrome, aniline blue, pentachrome, and Picrosirius red. Histomorphometric analysis is performed easily with either aniline blue or pentachrome stains, in which osteoid appears dark blue and yellow, respectively. Pixels per high powered field are calculated using the magic wand tool in Adobe Photoshop.

7. Representative Results

As both the calvarial and femoral defects are critical-sized, no significant healing should be expected without treatment with growth factors or exogenous stem cells.

In terms of surgical maneuvers, the muscle pouch dissection should be along fascial planes and thus minimal bleeding should be encountered. Even though the muscle pouch model is performed bilaterally, the mouse should be walking with ease on postoperative day 1. For the calvarial defect, bleeding is encountered but can be soaked with a Q-tip. Extreme care should be taken not to injure the underlying dura mater - as this will interfere with normal healing. For the FSD model, care is taken not to injure the major blood vessels so as not to cause excessive bleeding or the femoral nerve to prevent neurologic damage. Kirschner wires are drilled with gentle pressure so as not to damage the cortical bone in the process.

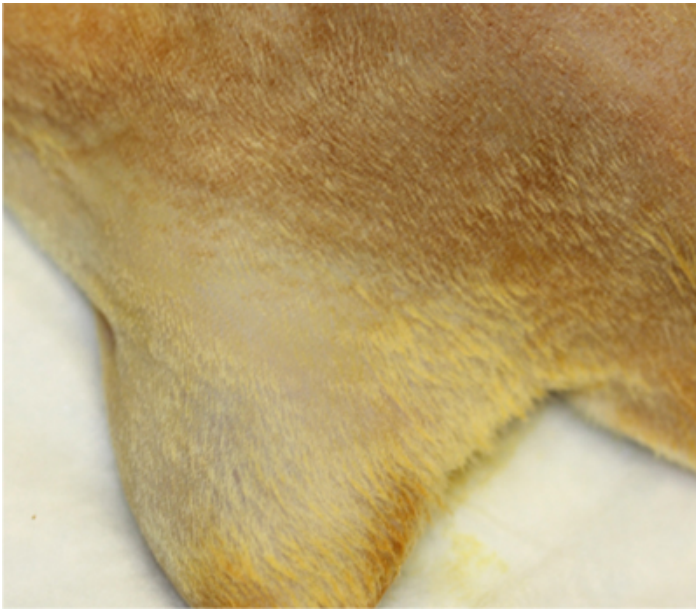


Figure 1. Preoperative preparation for Femoral Segmental Defect (FSD) in Athymic Rats. Male rats (12-14 weeks old) are anesthetized under isoflurane inhalation. The femur is scrubbed and prepped per standard protocol with betadine.



Figure 2. Surgical exposure for Femoral Segmental Defect (FSD) creation. A 27-30 mm longitudinal incision is made over the anterolateral aspect of the femur. The lateral aspect of the femoral shaft is then exposed by separating the vastus lateralis and biceps femoris muscles.

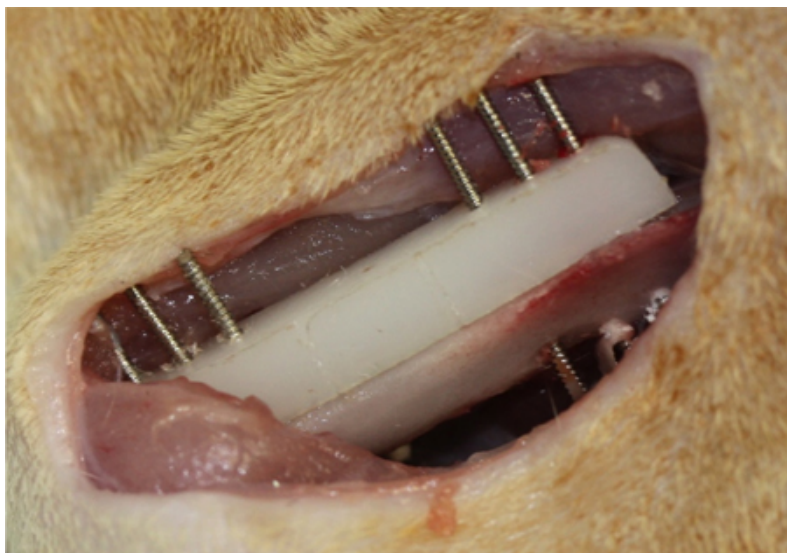


Figure 3. Fixation for Femoral Segmental Defect (FSD) creation. A polyethylene plate (length, 23 mm; width, 4 mm; height, 4 mm) is placed on the anterolateral surface of the femur. The plate contains six pre-drilled holes to accommodate 0.9 mm diameter threaded Kirschner wires. Taking the plate as a template, six threaded Kirschner wires are drilled through the plate and both cortices. Next, a 6 mm mid-diaphyseal defect is created. Once this is performed, a custom-made scaffold is directly inserted which exactly fits the defect site (not shown).

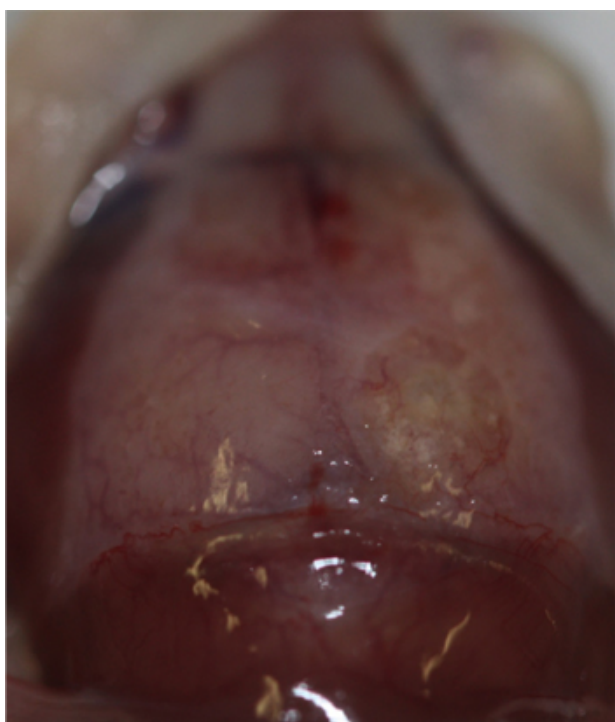


Figure 4. Example of Calvarial Defect Postoperative. A 4 mm, circular calvarial defect is created in the right parietal bone in athymic mice. Imaged here is a defect site implanted with PSCs at 8 weeks postoperative. Note the presence of new bone within the defect site.

Discussion

The isolation of PSCs is well described elsewhere¹⁻³, including a separately submitted JoVE publication specifically addressing PSC isolation protocols and methods. The specific purpose of this article is to describe and demonstrate 3 protocols for PSC *in vivo* application for bone formation/regeneration. The SCID mouse muscle pouch is a commonly described model for ectopic human bone formation⁷. Important differences exist between ectopic and orthotopic (defect) models for bone, including paracrine interaction with host bone-forming cells⁸ as well as an abundance of osteogenic signaling factors present in the skeletal defect microenvironment. Two defects are presented here, a calvarial defect⁸ and femoral segmental defect⁴. Both are well-documented to be critical sized (i.e. will not heal on their own).

Interesting differences exist between calvarial and femoral defects. First, the cell:cell interaction between xenografted PSCs and endogenous cells is very different. In terms of a calvarial defect, PSCs interact with the underlying dura mater (the outermost layer of the meninges), as well as those osteoblasts and periosteal cells circumscribing the defect site. Importantly, the interaction between implanted cells and surrounding osteoblasts⁸, or implanted cells and underlying dura (Levi *et al.*, in press) are critical for normal stem cell mediated osteogenesis to proceed. In terms of the femoral segmental defect (FSD), xenografted PSCs are exposed to a very different cell and cytokine environment. For example, the

FSD site is composed of the marrow and accompanying mesenchymal stem cells, as well as the endosteum, periosteum and long-bone osteoblasts. Theoretically, each cell has its own reaction to injury, and each may have cell:cell interactions with PSC xenografts.

Other clear differences exist between calvarial and femoral defects. The calvarial bones initially form through intramembranous ossification, while the long bones form through a cartilage intermediate (endochondral ossification). Moreover, the reparative process post-injury also mimics these developmental origins. Post-FSD, cartilage callus formation is observed, whereas no cartilage intermediate is formed within a parietal bone defect. Finally, the embryonic origin of the skull may differ from that of the long bones. The majority of the skull (including perivascular cells – pericytes – in the whole head region) is derived from the neural crest (mesectoderm), while the appendicular skeleton is of paraxial mesoderm derivation⁹. All these differences may result in significant differences in terms of PSC-mediated bone repair.

The use of PSCs has several benefits over traditional adipose-derived stromal cells (ASCs). PSCs do not require culture and are a purified cell population which does not include other stromal cells that do not participate in – and can even negatively regulate – osteogenic differentiation, such as endothelial cells¹⁰. In contrast, for example, clonal analyses of ASCs show that only a subpopulation are capable of undergoing osteogenic differentiation *in vitro*¹¹. Ultimately, skeletal tissue engineering efforts will likely incorporate an osteocompetent stem cell (such as PSCs) with exogenous growth factors and an osteoconductive scaffold (such as HA-PLGA used in the present methods) so as to best heal skeletal defects.

Disclosures

K.T, B.P., and C.S. are inventors of perivascular stem cell-related patents filed from UCLA. Drs. K.T. and C.S. are founders of Scarless Laboratories Inc. which sublicenses perivascular stem cell-related patents from the UC Regents. Dr. Chia Soo is also an officer of Scarless Laboratories, Inc.

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